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(54) [Title of the Invention] **A Method for the Determination of Low Specific Gravity Oxidized Lipoprotein**

(57) [Abstract]

[Objective] To provide a simple and rapid method of determining the quantity of low specific gravity lipoprotein that is useful as an indicator for early discovery of coronary artery diseases such as atheroma arteriosclerosis or groups of diseases preliminary to such conditions and that has been chemically modified by oxidation in plasma or serum that has been subjected to oxidation treatment.

[Structure] A method for the determination of oxidized LDL characterized in that plasma or serum is treated by oxidation and in that the low specific gravity lipoprotein present in the plasma (or serum) that has been oxidatively modified (hereafter abbreviated as LDL) is determined by an immunological determination method using antibodies that have specificity for oxidized LDL.

[Claims]

[Claim 1] A method for the determination of oxidized LDL characterized in that plasma or serum is treated by oxidation and in that the low specific gravity lipoprotein present in the plasma (or serum) that has been oxidatively modified (hereafter abbreviated as LDL) is determined by an immunological determination method using antibodies that have specificity for oxidized LDL.

[Claim 2] A reagent kit for determination of oxidized LDL which contains monoclonal antibodies that have specificity for oxidized LDL and an oxidizing agent.

[Claim 3] Monoclonal antibodies that have specificity for oxidized LDL.

[Claim 4] Monoclonal antibodies as described in Claim 3, which antibodies have specificity for oxidized LDL, are monoclonal antibodies that have the property that they do not bind with normal low specific gravity lipoproteins.

[Claim 5] Monoclonal antibodies as described in Claims 3 and 4, which antibodies that have specificity for oxidized LDL, have the property that they bind specifically with acetylated low specific gravity lipoproteins and malondialdehyde modified low specific gravity lipoproteins.

[Detailed Description of the Invention]

[0001]

[Field of industrial use] This invention relates to a method for the determination of low specific gravity lipoprotein that is useful as an indicator for early discovery of patients with coronary artery diseases such as atheroma arteriosclerosis or groups of diseases preliminary to such conditions and that has been chemically modified by oxidation in plasma (or serum) that has been subjected to oxidation treatment.

[0002]

[Prior art] In atheromatous arteriosclerosis, which is an arteriosclerotic disease, deposition of cholesterol or formation of plaque comprised of cholesterol and waste products in the tunica intima of arteries are seen.

[0003] For example, macrophages originating from monocytes, epithelial cells and scavenger receptors that have been discovered on vascular smooth muscles cells that have changed from the contractile type to the synthetic type are involved in the incorporation of cholesterol in the tunica intima of arteries. It is known that said scavenger receptors recognize and incorporate low specific gravity lipoprotein LDL that has been oxidized (chemical modified by oxidation) (hereafter abbreviated as oxidized LDL), for example, chemically modified LDL such as acetylated LDL and malondialdehyde modified LDL and LDL that has been chemically modified oxidatively by cultured epithelial cells, monocytes and smooth muscle cells.

[0004] It is believed that the cholesterol that accumulates in atheromatous arteriosclerosis originates primarily from oxidized LDL that has been incorporated through the agency of scavenger receptors. For this reason, it can be presumed that determination of oxidized LDL in plasma or serum is useful as an indicator for early discovery of coronary artery disease originating in atheromatous arteriosclerosis and groups preliminary to these diseases.

[0005] However, oxidized LDL has the property that it replenishes scavenger receptors and the property that it is extremely easily metabolized. Moreover, there are many instances in which exogenous antioxidants such as tocopherol (vitamin E), ascorbic acid and β-carotene are ordinarily present in the blood flow so that LDL is protected from oxidative chemical modification. Consequently, it is the present state of affairs that the quantity of oxidized LDL in the blood flow (or in the plasma or in the serum) is extremely small and is essentially undetectable.

[0006] On the other hand, instead of finding the quantity of oxidized LDL in the blood flow (or in the plasma or in the serum), a method for studying the capacity for LDL to undergo oxidation easily has been proposed by Estabana [phonetic]* et al. in which LDL is separated from plasma or serum by ultracentrifugation , cupric ions or 2,2'-azobis(2-amidinopropane) dihydrochloride is added to the purified LDL that is obtained and the quantity of conjugated diene that is produced as incubation is carried out at a fixed temperature is determined as changes in absorbance (OD 234) (Methods in Enzymology, Vol. 233, pp. 425-441: 1994). Specifically, it is thought that the capacity for LDL to undergo oxidation reflects the quantity of oxidized LDL that can be produced in the blood flow. For this reason, its use as an indicator for early discovery coronary artery diseases such as atheromatous arteriosclerosis and groups preliminary to these diseases is being studied.

[0007] However, these methods for studying the capacity of LDL for undergoing oxidation present various problems in that the procedures are complex, in that it is difficult to handle large volume samples and in that the values that are obtained do not necessarily reflect the capacity for LDL to undergo oxidation in the blood flow (or in plasma or in serum) even though they serve to estimate the capacity of purified LDL to undergo oxidiation.

[0008] Other methods that have been developed as indicators for discovering coronary artery disease or groups preliminary to such diseases include a procedure in which the quantity of antigen in the serum that reacts with monoclonal antibodies to antigen associated with human atheroma is determined (Japanese Patent Application Early Disclosure No. 4-159300 [1992]) and a method in which the quantity of antigen in serum that reacts with monoclonal antibodies to oxidized LDL is determined (WO 94/23302). However, no significant differences were found in the quantities of antigen that can be determined by these methods between normal individuals and patients with coronary artery disease. For this reason, it is doubtful whether the values determined for antigen are sufficiently useful to serve as indicators for discovering patients with coronary artery diseases.

[0009]
[Objective of the invention] This invention, which was developed in view of the aforementioned circumstances, has the objective of providing a simple and rapid method of determining the quantity of low specific gravity lipoprotein that is useful as an indicator for early discovery of coronary artery diseases such as atheroma arteriosclerosis or groups of diseases preliminary to such conditions and that has been chemically modified by oxidation in plasma or serum that has been subjected to oxidation treatment.

[0010]
[Structure of the invention] This invention is the invention of a method for the determination of oxidized LDL characterized in that plasma or serum is treated by oxidation and in that the low specific gravity lipoprotein present in the plasma (or serum) that has been oxidatively modified (hereafter abbreviated as LDL) is determined by an immunological determination method using antibodies that have specificity for oxidized LDL.

[0011] This invention is the invention of a kit for determination of oxidized LDL which contains monoclonal antibodies that have specificity for oxidized LDL and an oxidizing agent.

[0012] This invention is the invention of monoclonal antibodies that have specificity for oxidized LDL

[0013] Specifically, the inventors, in the course of conducting intensive research on determination of the quantity of oxidized LDL in plasma or serum, which they believed would be useful as an indicator for early discovery of coronary artery disease and groups preliminary to such diseases, discovered that there was essentially no difference between the quantities of oxidized LDL actually present in the plasma (or serum) of normal persons and patients with coronary artery disease, in other words, that early discovery of coronary artery disease and groups [of diseases] which are precursors to such diseases

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* [Translator's Note: Transliterated phonetically from the Japanese. As such, the spelling may differ from other transliterations.]

was difficult using such findings as an indicator. Accordingly, the inventors conducted further intensive research, and, as a result, discovered that the quantity of oxidized LDL (i.e., the quantity of oxidized LDL including oxidized LDL originating in easily oxidizable LDL) can be determined simply and with high precision when the plasma or serum that is the object of determination is subjected to an oxidation treatment in advance with an oxidizing agent and a water-soluble azo polymerization initiator, after which the oxidized LDL in said plasma (or serum) is determined by an immunological determination method using antibodies to oxidized LDL. Further, when the quantities of oxidized LDL in said plasma or serum obtained in this way were compared between normal individuals and patients with coronary artery disease, it was discovered that there was a significant difference between them, in other words, that the quantities of oxidized LDL found in this way were useful as indicators for early discovery of patients with coronary artery disease and groups [of diseases] which are precursors to these diseases. As a result, this invention was perfected.

[0014] There are no particular limitations on the reagent that is used for oxidation treatment of plasma (or serum) as long as it has the capacity to obtain easily oxidizable LDL in plasma or serum as oxidized LDL and does not affect the determination system (immunological determination system) after treatment. For example, it may be an oxidizing agent that contains metal ions such as ferrous ions or a water-soluble azo polymerization initiator. There are no particular limitations on the aforementioned oxidizing agents that contain metal ions. For example, they may include copper sulfate, cuprous salts such as cuprous chloride and ferrous salts such as ferrous chloride, ferrous sulfate and ferrous nitrate. There are no particular limitations on the concentrations in which they are used as long as it is a concentration at which easily oxidizable LDL can be oxidized. Although there are some differences depending on the type of oxidizing agent, the concentration in the solution when oxidation treatment is performed may ordinarily be selected from a range of catalytic quantity to 2000 μmol/liter, and, preferably, 100 to 500 μmol/liters.

[0015] There are no particular limitations on the water-soluble azo polymerization initiator that can be used in this invention as long as it is water-soluble, has the capacity to obtain easily oxidizable LDL in plasma or serum as oxidized LDL and does not affect the determination system (immunological determination system) after treatment. For example, they can be commercial water-soluble azo polymerization initiators including azoamidine salts such as 2,2'azobis(2-amidinopropane) dihydrochloride and 2,2'-azobis[2-(2-imidazoline-2-ol)propane] dihydrochloride, azoamides such as, for example, 2,2'-azobis(2-methylpropionamide) 2-hydrate, and 4,4'-azobis(4-cyanovaleric acid) and 2,2'-azobis(2-methylproipionamideooxime). There are no particular limitations on the concentrations in which they are used as long as the concentrations are at the level at which easily oxidizable LDL can be obtained as oxidized LDL. Although there are some differences depending on the type of azo polymerization initiator, the concentration in the solution at the time the treatment is performed can ordinarily be selected from a range of 0.1 to 20 mM, and, preferably, of 0.5 to 10 mM.

[0016] There are no particular limitations on the antibodies having specificity to oxidized LDL that are used in this invention as long as they have the property that they can combine specifically with LDL that has been subjected to oxidative chemical modification by the reagents that are used in this invention for oxidative treatment of serum. It goes without saying, of course, that this objective cannot be achieved in the method of determination of this invention if at least one type of antibody having the property of specificity to oxidized LDL and the property of not reacting with normal LDL (does not combine with normal LDL) is not used. Specifically, for example, when the quantity of oxidized LDL is determined by the sandwich enzyme immunological determination method making use of the principles of noncompetitive methods, the quantity of oxidized LDL cannot be determined precisely if either the antibodies that are made into a solid phase with the surfaces of a carrier such as, for example, a microplate, glass beads, plastic beads or latex or enzyme-labeled antibodies are not antibodies having the property that they have specificity for oxidized LDL and that they do not react with normal LDL.

[0017] Further, the antibodies having specificity for oxidized LDL of the invention can be prepared by immunizing animals such as, for example, horses, cows, rabbits, goats, rats and mice, by standard methods, for example by the method described in "Introduction to Immunological Testing," by Naoshi Matsuhashi, et al. (Gakkai Shuppan Center (Ltd.), 1981). They can also be obtained from hybridomas that produce antibodies having specificity for oxidized LDL that are obtained by standard methods, i.e., by fusing cells from mouse tumor lines with mouse spleen cells immunized in advance with oxidized LDL in accordance with the cell fusion method established by Keller [phonetic] and Milstein [phonetic] (Nature, Vol. 256, p. 495, 1975). These polyclonal antibodies and monoclonal antibodies can be used individually or in combination.

[0018] Particularly desirable antibodies having specificity for oxidized LDL of this invention are monoclonal antibodies that have the property that they combine specifically with oxidized LDL, acetylated LDL and malondialdehyde modified LDL. When determination of oxidized LDL (oxidized LDL in plasma or serum treated with the aforementioned oxidizing agent) is performed using these antibodies, the differences in the quantities of oxidized LDL in normal subjects and patients with coronary artery disease can be differentiated more precisely.

[0019] Specifically, in consideration of the fact that cholesterol that has accumulated in the sites of lesions in atheromatous arteriosclerosis originates from LDL that has been bound to and incorporated in the scavenger receptors or macrophages and that has been modified in some form (for example, oxidized LDL, acetylated LDL, malondialdehyde modified LDL and LDL that has undergone oxidative chemical modification by cultured epithelial cells, monocytes and smooth muscle cells), if determination of oxidized LDL can be made using antibodies that recognize the characteristic structures common to these modified forms of LDL, the difference in the quantity of oxidized LDL that occurs in normal individuals and patients with coronary artery disease as a result of oxidation treatment (i.e., the difference in the quantities of easily oxidizable LDL) can be differentiated more precisely.

[0020] The monoclonal antibodies of this invention that have the property of combining specifically with oxidized LDL, acetylated LDL and malondialdehyde modified LDL can, for example, be obtained easily as described below.

[0021] Specifically, cells from a mouse tumor line and spleen cells of mice that have been immunized in advance with a mixture of oxidized LDL, acetylated LDL and malondialdehyde modified LDL are fused following the cell fusion method established by Keller and Milstein (Nature, Vol. 256, page 495, 1975), with a hybridoma being obtained. Next, the antibodies that said hybridoma produces and that have specificity for oxidized LDL, acetylated LDL and malondialdehyde modified LDL may be collected as described in the report.

[0022] The method of this invention may be executed, for example, as described below. Specifically, plasma or serum and, for example, physiological saline solution or buffer solution that contains a suitable concentration of oxidizing agent are mixed so that the oxidizing agent is in the concentration indicated above and the mixture is immediately stirred, after which it is incubated at 20 to 50°C, preferably, at 25 to 40°C and for 10 minutes to 24 hours, and, preferably, for 6 to 16 hours. The product is taken as the test sample. The quantity of oxidizing LDL in said plasma or serum may then be determined by a standard method such as an immunological determination method in which antibodies having the property described above are used, for example, the enzyme immunoassay method (EIA; reference: The Enzyme Immunoassay Method (3rd edition), Eiji Ishikawa, Editor, 31-54 (1987), published by Igaku Shoin (Ltd.)), the radioimmunoassay method (RIA, reference: S.A. Berson, R.S. Yallow, J. Clin. Inves., vol. 38, 1996 (1959)), the turbidimetric immunoassay method (TIA, reference: Sakurabayashi, et al, Nippon Rinsho [The Japanese Journal of Clinical Medicine], Vol. 42, pages 1214-1220 (1984)) and the immunological specific filtration method (reference: K. Hoffken, C. G. Schmidt, "Methods in Enzymology," vol. 74, page 628 (1981)).

[0023] The degree of oxidation of easily oxidizable LDL differs somewhat depending on the type of oxidizing agent and the azo polymerization initiator, their concentrations during oxidation treatment and the temperature and duration of incubation. When the quantity of easily oxidizable LDL that is

obtained is used as the indicator for early discovery of coronary artery disease or groups preliminary to disease, it goes without saying that the conditions described above must be fixed. For example, in order to fix incubation time (oxidation treatment time), a treatment may be carried out in which an antioxidant such as, for example, ethylenediamine tetraacetic acid or ascorbic acid is added to the reaction solution after incubation for a fixed time in an excess quantity that does not affect subsequent determination systems.

[0024] The kit for determination of easily oxidizable LDL of this invention is used for the purpose of determining the aforementioned quantity of easily oxidizable LDL and contains monoclonal antibodies that have specificity for oxidized LDL and an oxidizing agent. Desirable modes of the various component conditions are described above for specific examples. We shall now describe this invention in further detail by presenting reference examples and examples. However, this invention is not limited in any way by them.

[0025]

[Examples]

Reference Example 1. Method of Preparation of Modified LDL

An LDL fraction was obtained from fresh human serum by the ultracentrifugation method (Continuing Lectures in Biochemical Experimentation 3, compiled by the Japanese Biochemistry Society, page 595, 1986, published by Tokyo Kagaku Dojin (Ltd.)) and acetylated LDL, malondialdehyde modified LDL and oxidized LDL were obtained by the following methods using it as the raw material.

a) Acetylated LDL

It was prepared as described below by a partially modified version of the method of Basu et al. (Basu et al., Proc. Natl. Acad. Sci., USA, Vol. 73, pp. 3178-3182, 1976). Specifically, 0.5 ml of saturated sodium acetate solution was added to 0.5 ml of LDL (5 mg/ml) over ice, 7 μl of acetic anhydride was added four times at intervals of 15 minutes as the mixture was being stirred with a stirring rod and stirring was continued for another 30 minutes, after which dialysis was performed for 2 days against a solution of 0.15M NaCl and 2mM EDTA-3Na. The LDL that was obtained was taken to be acetylated LDL.

b) Malondialdehyde modified LDL

It was prepared as described below following the method of Fogelman et al. (Proc. Natl. Acad. Sci., USA, Vol. 77, pp. 2214-2218, 1980). Specifically, 88 μl of malonaldehyde bis(dimethylacetal) and 400 μl of purified water were mixed, the mixture was maintained at 37°C, 12 μl of 4N hydrochloric acid solution was added and the materials were incubated for 10 minutes at 37°C. Immediately thereafter, 130 μl of 2N sodium hydroxide solution and 350 μl of purified water were added and mixed, with an 0.5M malondialdehyde solution (pH 7.0) being obtained. 0.5 ml of this solution and 0.5 ml of LDL (5 mg/ml) were mixed and the mixture was incubated for 3 hours at 37°C. The product was taken as malondialdehyde modified LDL.

c) Oxidized LDL

Copper sulfate was added to 1 ml of a 200 μg/ml LDL solution to give 10 μM and this mixture was incubated for 4 hours at 37°C. Next, 100 μl of 22 mM EDTA-3Na solution was added to stop the reaction. The substance that was obtained was taken as oxidized LDL.

Example 1. Preparation of Monoclonal Antibodies

Anti-oxidized LDL monoclonal antibodies were prepared as described below in accordance with a known cell fusion technique using an equimolar mixed solution of the acetylated LDL, the malondialdehyde modified LDL and the oxidized LDL prepared in Reference Example 1 as the immunogen. Specifically, the equimolar mixed solution of acetylated LDL, malondialdehyde modified LDL and oxidized LDL was used as the antigen solution, a solution prepared in accordance with the standard procedural method of the Libby [phonetic] adjuvant system (manufactured by Libby Immunochem Research Company) was used

as the immunogen and they were administered intraperitoneally three times at intervals of two weeks to Balb/c mice so that total volume of acetylated LDL, malondialdehyde modified LDL and oxidized LDL was 30 μg/animal. One week after the final immunization, a preparation obtained by diluting an equimolar mixed solution of acetylated LDL, malondialdehyde modified LDL and oxidized LDL with physiological saline solution was injected intraperitoneally into mice to give 30 μg/animal. Three days after this injection was given, the spleen of the mouse was excised and the spleen was thoroughly disentangled using sterilized frosted glass, after which it was suspended in Daigo [phonetic] culture medium (manufactured by Nihon Seiyaku (Ltd.)), centrifugation treatment was repeated several times and it was thoroughly washed. 1.5×10^8 of these washed spleen cells and 1.5×10^7 of mouse myeloma cells (P3-NS-1-Ag4(NS-1)) that had been thoroughly washed with Daigo T culture medium were collected in a test tube and mixed, after which they were spread on the bottom of the test tube. 1 ml of polyethylene glycol 6000 (manufactured by Waki Junyaku Kogyo (Ltd.)) solution made to 50 w/v % was poured in gently and thoroughly mixed and a cell fusion reaction was carried out for one minute, after which 11 ml of Daigo T culture medium was added slowly to dilute the PEG and the cell fusion reaction was stopped. The cell suspension that was obtained was centrifuged for 5 minutes at 1500 revolutions, the supernatant was removed and the cells were suspended in 100 ml of Daigo T culture medium containing 10% bovine fetal serum. This suspension was injected in amounts of 0.1 ml each into each well of 96-hole microplate and the materials were incubated at 37°C in the presence 5% CO_2. After incubation for 24 hours, HAT culture medium of twice the ordinary concentration was injected in amounts of 0.1 ml into each well and incubation was carried out for about 48 hours. The culture supernatant in each well was replaced by ordinary HAT culture medium. One week after fusion, amounts of 0.1 ml of culture supernatant of each well were removed and amounts of 0.1 ml of HT culture medium were added. This procedure was also carried out on the next day. Ten days after the cell fusion reaction, the antibody titer of the culture supernatant was studied, cells of wells exhibiting strong antibody activity were cloned by the limiting dilution method and clones that produce antibodies having the properties indicated in Table 1 below were obtained. In Table 1, +++, ++, + and - indicate the reactivity of the various types of antibodies with the various types of LDL, +++ indicating an extremely strong reaction, ++ indicating a strong reaction, + indicating a weak reaction and - indicating that a reaction did not occur.

[0026]

Table 1

Clone No.	Subclasses of antibodies that are produced	Reactivity of antibodies with various types of modified LDL*			
		Normal	Acetyl	MDA	Oxidized
LD1 - A2	G_1	-	+++	+++	++
LD1 - E3	G_1	++	++	++	++
LD2 - G11	G_{2b}	-	++	++	+
LD2 - D6	G_1	+	+	+	+
LD3 - B5	G_1	+++	+++	+++	+++
LD3 - E2	G_1	+++	++	++	+
LD3 - H2	G_1	+	++	++	+
LD4 - B11	G_1	+	++	++	++
LD4 - H8	G_{2a}	+	+++	+++	+
LD4 - C8	G_1	+	++	++	+
LD5 - B7	G_1	-	-	+++	+

* Normal: normal LDL; Acetyl: acetylated LDL; MDA: malondialdehyde modified LDL; Oxidized: oxidized LDL

Of the aforementioned clones, LD1-A2 and LD3-B5 are deposited in the Life Industrial Technology Institute of the Agency of Industrial Science and Technology of the Ministry of International Trade and Industry. The Deposition Numbers and Deposition Dates are as follows.

Clone LD1-A2: Deposition Number: FERM P-14910. Deposition Date: 28 April 1995.

Clone LD3-B5: Deposition Number: FERM P-14911. Deposition Date: 28 April 1995.

[0027] Example 2. Study of Reactivity of Various Types of Lipoproteins

A study was made as described below to determine whether the method of determination of oxidized LDL using monoclonal antibodies of this invention exhibits reactivity with various types of lipoproteins. In this study, we used monoclonal antibodies produced by the clone LD1-A2 that do not react with normal LDL having specificity for acetylated LDL, malonaldehyde modified LDL and oxidized LDL in solid phase antibodies and [we used] monoclonal antibodies produced by the clone LD3-B5 that react widely with various modified LDL in secondary antibodies, which antibodies were labeled with peroxidase (manufactured by Toyobo (Ltd.)) by a standard method (Ishikawa, E., et al., J. Immunoassay, Vol. 4, pages 209-327, 1983). Specifically, 50 μl of a 20 μgAb/ml solution of clone LD1-A2 monoclonal antibodies was injected and a fixing treatment was carried out for 30 hours at 37°C, after which 100 μl buffer solution for the primary reaction [100 mM phosphate buffer solution (pH 7.4) containing 25 (V/V) % of Block Ace [phonetic] and 0.1 (W/V) % of ethylenediaminetetracetic acid · disodium salt] was introduced into each well of 96 hole microplate that had been subjected to blocking treatment with 25 (V/V) % Block Ace [phonetic] (manufactured by Snow Brand Milk Products Co. (Ltd.)) solution, incubation was performed for 6 hours at 37°C in the presence of 250 μM copper sulfate and amounts of 10 μl of various lipoprotein solutions that had been subjected to oxidation treatment were added. These materials were incubated for 2 hours at 37°C, after which they were washed three times with 300 μl of phosphate buffer solution (pH 7.4) – physiological saline solution containing 0.05% Tween 20 (Aldrich) (hereafter, PBS-Tween). Amounts of 50 μl of buffer solution for the secondary reaction (PBS-Tween containing 25 (W/V) % of Block Ace) containing peroxidase labeled clone LD3-B5 produced monoclonal antibodies to give a concentration of 1.5×10^{-3} at OD 403 were introduced into each well and incubation was performed for 2 hours at 37°C. Next, the materials were washed three times with 300 μl of PBS-Tween, after which amounts of 50 μl of Macklebine [phonetic] buffer solution (pH 4.8) containing 14.4 mM of o-phenylenediamine were added to each well and an enzyme reaction was carried out for 30 minutes at room temperature. Amounts of 50 μl of 3N sulfuric acid were then added to each well and the reaction was stopped. Absorbance (OD 490-650) of each well was determined with a microplate reader UV max (manufactured by Molecular Devices Company) set to the conditions λ1 = 490 nm, λ2 = 650 nm and two wavelength end point determination by a SOFTmax-J (Ver. 2.2, manufactured by Wako Junyaku Kogyo (Ltd.)). A study was made of reactivity with various lipoproteins that had undergone oxidation treatment.

(Results) The results are shown in Table 2. In Table 2, +++ indicates that the absorbance that was obtained was greater than 0.5, ++ indicates that the absorbance obtained was 0.25 to 0.5, + indicates that the absorbance obtained was 0.1 to 0.25 and – indicates that the absorbance obtained was less than 0.1.

[0028]

Table 2

Types of lipoproteins	Reactivity
Normal LDL	–
Acetylated LDL	+++
Malondialdehyde modified LDL	+++
Malondialdehyde modified LDL ($NaCNBH_3$ reduced)	+++
Acetylated HDL	–
Acetylated VLDL	+
Malondialdehyde modified HDL	–
Malondialdehyde modified VLDL	++
Saccharified LDL	+
Oxidized LDL	++

From the results in Table 2, it can be seen that determinations could be made of the quantity of oxidized LDL, i.e., of the quantity of oxidized LDL originating from easily oxidizable LDL, without producing determination errors attributable to normal LDL and various types of HDL by using the determination method in which the monoclonal antibodies of this invention were used.

[0029] Example 3. Study of the degree of oxidation of LDL and of the reactivity of monoclonal activities originating from the clone LD1-A2

Studies were conducted of the degree of oxidation of LDL and of the reactivity of monoclonal antibodies originating from the clone LD1-A2. The degree of oxidation of LDL was studied using as indicators the quantity thiobarbituric acid substance (TBARS) and the quantity of lipid peroxide (LPO), which indicated degree of oxidation.

(Preparation of test materials) Copper sulfate was added to a 200 μg/ml solution of LDL prepared by the ultracentrifugation method using fresh human serum as the raw material (Continuing Lectures in Biochemical Experimentation 3, compiled by the Japanese Biochemistry Society, page 595, 1986, published by Tokyo Kagaku Dojin (Ltd.)) to give 10 μM and incubation was performed for a specified time at 37°C, after which a 22 mM EDTA-3Na solution of one-tenth the volume of the reaction solution was added to stop the reaction. The product was used as the test material.

(Determination of the quantity of TBARS) 1 ml of 20 w/v% trichloroacetic acid was added to 200 μl of the specified test material and the mixture was stirred, after which 2 ml of 0.67% thiobarbituric acid solution was added and the solution was heated for 20 minutes in a boiling water bath and then allowed to cool. The reaction solution was centrifuged for 15 minutes at 3000 rpm and the absorbance (535 nm) of the supernatant that was obtained was determined. Absorbance was determined for the test material using physiological saline solution as the control. The absorbance that was obtained was converted for the quantity of malondialdehyde (nmol/mg protein) taking the molecular absorbance coefficient of malondialdehyde as $1.56 \times 10^5 M^{-1}cm^{-1}$.

(Determination of quantity of LPO) Determination of LPO was performed using a commercial lipid peroxide determination reagent "Determiner LPO" (manufactured by Kyowa Medix (Ltd.)). The determination procedure was performed by the standard method described in the product description included with the kit.

(Study of the reactivity of monoclonal antibodies originating from the clone LD1-A2) The quantity of oxidized LDL in the specified test materials was determined from the absorbance (OD 490-650) using the same reagent and the same procedure as in Example 2 except that 10 μl of the test material obtained above was used instead of 10 μl of various types of lipoprotein solution that had undergone oxidation treatment.

(Results) The results are shown in Figure 1. In Figure 1, ▲ indicates the absorbance (quantity of oxidized LDL in the sample that had undergone oxidation treatment) obtained for the specified test material, O indicates the quantity of TBARS (nmol/mg protein) obtained for the specified test material and □ indicates the quantity of LPO (μmol/mg protein) obtained for the □ specified test material. From Figure 1, it can be seen that oxidized LDL under these conditions can be detected by the method of this invention from the fact that absorbance (OD 490-650) begins to rise when incubation time of the test material exceeds 150 minutes. Further, the quantity of TBARS and the quantity of LPO are the indicators of the degree of oxidation of LDL. However, because there are essentially no changes in them once incubation time exceeds 180 minutes, it can be concluded that oxidative chemical modification of LDL is concluded at about this point. On the basis of the foregoing results, it can be concluded that monoclonal antibodies originating from the clone LD1-A2 react with LDL that has undergone sufficient oxidative chemical modification but that they do not react with LDL in which oxidative chemical modification has not proceeded to that extent (LDL in the initial stage of the oxidation reaction). Monoclonal antibodies developed by Bumol [phonetic] et al. that react with antigen associated with human atheroma (Japanese Patent Application Early Disclosure No. 4-159300 [1992] have the property of reacting with LDL in the initial stage of oxidation reactions, for which reason they have properties different from those of the monoclonal antibodies of this invention.

[0030] Example 4. Study of Copper Ion Concentrations During Oxidation Treatment

(1) Preparation of serum samples that have undergone oxidation treatment

50 μl of specified fresh serum was introduced into a tube and 50 μl of 0.9% NaCl solution containing a specified concentration of copper sulfate was mixed with it. This mixture was introduced into a constant temperature tank set at 37°C and allowed to stand for 16 hours shielded from light. Following that, 10 μl of 2.2 mM ethylenediamine tetracetic acid · trisodium salt was added to the reaction solution to stop the reaction and a serum sample that had undergone oxidation treatment was obtained.

(2) Determination of the quantity of oxidized LDL in samples that have undergone oxidation treatment

The quantity of oxidized LDL that undergoes oxidation treatment was found by determining the absorbance using the same reagent and by the same procedure as in Example 2 except that 10 μl of the serum sample obtained in (1) that had undergone oxidation treatment was used instead of the 10 μl of various types of lipoprotein solutions that had undergone oxidation treatment. The results are shown in Figure 2. In Figure 2, Δ shows the results when serum originating from normal persons was used and ○ and □ show the results when serum originating from patients hospitalized for concentrated treatment for coronary artery disease (hereafter abbreviated as CCU serum) was used. The copper ion concentration on the horizontal axis in Figure 2 indicates the copper ion concentration (μm) at the time of the oxidation treatment reaction. From Figure 2, it can be seen that, with CCU serum, elevations of absorbance were seen accompanying elevation of copper ion concentration for oxidation treatment. By contrast, with serum of normal persons, elevations of absorbance were not seen up to copper ion concentrations during oxidation treatment of up to 260 μM. In other words, the copper ion concentration at the time of the oxidation treatment reaction should be 160 to 260 μM.

[0031] Example 5

The quantity of oxidized LDL in various types of serum samples that have undergone oxidation treatment was found by determining the absorbance using the same reagents and by the same procedure as in Example 2 except that 10 μl of a substance obtained by incubating 17 samples of CCU serum and 5 samples of serum of normal persons for 6 hours at 37°C in the presence of 250 μM copper sulfate and subjecting them to oxidation treatment was used instead of 10 μl of various types of lipoprotein solution that had undergone oxidation treatment. This absorbance was applied to a calibration curve indicating the relationship between the quantity of oxidized LDL and absorbance that was prepared using a malondialdehyde modified LDL solution of concentrations specified in advance as the LDL standard solution and using the same reagent and procedures as described above and the quantity of oxidized LDL (μg/ml) in various samples that had undergone oxidation treatment was found. The results are shown in Figure 3 and Table 3.

[0032] Comparative Example 1

The quantity of oxidized LDL in various serum samples was found by determining absorbance using the same reagent and by the same procedures as on Example 2 except that amounts of 10 μl of the 17 CCU serum samples and 5 samples of serum of normal persons used in Example 5 were used instead of 10 μl of various lipoprotein solution that have undergone oxidation treatment. This absorbance was applied to a calibration curve indicating the relationship between the quantity of oxidized LDL and absorbance that was prepared using a malondialdehyde modified LDL solution of concentrations specified in advance as the LDL standard solution and using the same reagent and procedures as described above and the quantity of oxidized LDL (μg/ml) in various samples that had undergone oxidation treatment was found. The results are shown in Figure 3 and Table 3.

[0033]

Table 3

Sample No.	Serum of normal persons		CCU serum	
	No oxidation treatment	Oxidation treatment	No oxidation treatment	Oxidation treatment
	Absorbance			
1	0.032	0.049	0.043	0.947
2	0.041	0.050	0.037	0.951
3	0.026	0.032	0.039	0.906
4	0.026	0.038	0.029	0.119
5	0.025	0.049	0.042	0.344
6	-	-	0.033	0.258
7	-	-	0.060	1.696
8	-	-	0.026	1.326
9	-	-	0.034	0.262
10	-	-	0.076	1.064
11	-	-	0.032	0.405
12	-	-	0.036	0.373
13	-	-	0.032	0.230
14	-	-	0.035	0.490
15	-	-	0.029	0.583
16	-	-	0.026	0.219
17	-	-	0.028	0.563
Average values	0.030	0.044	0.037	0.632

As can be seen from Figure 3 and Table 3, there was essentially no difference between the quantity of natural oxidized LDL in CCU serum and that in the serum of normal persons; in other words, the quantity of natural oxidized LDL cannot be used as an indicator for the purpose of early discovery of coronary artery disease such as atheromatous arteriosclerosis and groups [of diseases] which are precursors to these diseases. By contrast, when the quantity of oxidized LDL produced by oxidation treatment (the quantity of easily oxidizable LDL: the quantity obtained by subtracting the quantity of natural oxidized LDL from the quantity of oxidized LDL after the oxidation treatment) was compared, it can be seen from the results shown in Figure 3 and Table 3 that significantly higher values were shown for the quantity of easily oxidizable LDL in CCU serum than for that in the serum of normal persons, in other words, that the quantity of easily oxidizable LDL is useful as an indicator for early discovery of coronary artery diseases such as atheromatous arteriosclerosis and groups [of diseases] preliminary to such diseases. From the results shown in Figure 3 and Table 3, it can further be seen that the quantity of oxidized LDL in serum after oxidation treatment is also useful as an indicator for early discovery of coronary artery diseases such as atheromatous arteriosclerosis and groups [of diseases] preliminary to such diseases.

[0034] Example 6

The quantity of oxidized LDL in various serum samples was found by determining absorbance using the same reagent and by the same procedures as on Example 2 except that amounts of 10 μl of test materials obtained by incubating 40 samples of CCU serum and 10 samples of serum of normal persons for 6 hours at 37°C in the presence of 250 μM of copper sulfate and by performing oxidation treatment were used instead of 10 μl of various lipoprotein solution that have undergone oxidation treatment. This absorbance was applied to a calibration curve indicating the relationship between the quantity of oxidized LDL and absorbance that was prepared using a malondialdehyde modified LDL solution of concentrations specified in advance as the LDL standard solution and using the same reagent and procedures as described above and the quantity of oxidized LDL (μg/ml) in various samples that had undergone oxidation treatment was found. The results are shown in Figure 4. From Figure 4 it can clearly be seen that significantly higher values were found for oxidized LDL in CCU serum that had undergone oxidation treatment than for the serum of normal persons

that had undergone oxidation treatment. In other words, the quantity of oxidized LDL determined by this method is useful as an indicator for early discovery of coronary artery diseases such as atheromatous arteriosclerosis and groups which are precursors to such diseases.

[0035] Example 7

The quantity of oxidized LDL in serum samples treated with V-50 was found by determination of absorbance using the same reagent and by the same procedure as in Example 2 except that amounts of 10 μl of test materials obtained by mixing 20 samples of CCU serum and 5 samples of serum of normal persons with 0.9% sodium chloride solution containing 10 mM of 2,2'azobis(2-amidinopropane) · dihydrochloride (brand name: V-50, manufactured by Wako Junyaku Kogyo (Ltd.)) in a ratio of 1 : 1 and then incubating them for 6 hours at 37° instead of 10 μl of various lipoprotein solutions that had undergone oxidation treatment. This absorbance was applied to a calibration curve indicating the relationship between the quantity of oxidized LDL and absorbance that was prepared using a malondialdehyde modified LDL solution of concentrations specified in advance as the LDL standard solution and using the same reagent and procedures as described above and the quantity of oxidized LDL (μg/ml) in various samples that had undergone V-50 treatment was determined. The results are shown in Figure 5. As can be clearly seen from Figure 5, significantly higher values were found for the quantity of oxidized LDL in CCU serum than for that in the serum of normal persons when treatment was performed with V-50, which is a water-soluble azo polymerization initiator. In other words, the quantity of oxidized LDL determined by this method is useful as an indicator for early discovery of coronary artery diseases such as atheromatous arteriosclerosis and groups which are precursors to such diseases. In addition, the same serum samples were used and the quantity of oxidized LDL in the serum samples was found by determining absorbance using the same reagent and by the same procedures as in Example 2 except that amounts of 10 μl of test materials obtained by mixing the serum samples with 0.9% sodium chloride solution containing 500 μm of copper sulfate in a ratio of 1 : 1 and then incubating then for 6 hours at 37°C were used instead of 10 μl of various lipoproteins that had undergone oxidation treatment. The absorbance was applied to a calibration curve indicating the relationship between the quantity of oxidized LDL and absorbance that was prepared using a malondialdehyde modified LDL solution of concentrations specified in advance as the LDL standard solution and using the same reagent and procedures as described above and the quantity of oxidized LDL (μg/ml) in various samples that had undergone oxidation treatment was found. Figure 6 shows the correlation between the quantity of oxidized LDL that was obtained and the quantity of oxidized LDL obtained when treatment was performed with V-50 (correlation coefficient: 0.861). From Figure 6 it can clearly be seen that the quantity of oxidized LDL in serum can be determined to the same degree both when treatment is performed with copper sulfate and when treatment is performed with V-50.

[0036]

[Effect of the invention] As described above, this invention is an invention that provides a method for the rapid and simple determination of the quantity of oxidized LDL in plasma or serum that has undergone oxidation treatment and that the quantity of oxidized LDL found by the method of this invention is useful as an indicator for early discovery of coronary artery diseases such as atheromatous arteriosclerosis and groups which are precursors to such diseases. Therefore, it is an invention that makes a significant contribution to this industry.

[Brief Explanation of the Figures]

[Figure 1] This is a graph that shows the relationship that was obtained in Example 3 between the degree of oxidation of low specific gravity lipoprotein (LDL) and the degree of detection by the method of this invention when oxidation treatment was performed for specified times.

[Figure 2] This is a graph showing the relationship that was obtained in Example 4 between the concentration of copper ions and LDL that had undergone oxidative chemical modification (hereafter abbreviated as oxidized LDL) that was detected.

[Figure 3] This is a graph that shows the quantity of oxidized LDL in serum originating in patients hospitalized for treatment of coronary artery disease (hereafter abbreviated as CCU serum) and in the serum of normal persons that had undergone oxidation treatment obtained in Example 4 and the quantity of oxidized LDL in CCU serum and the serum of normal persons that had not undergone oxidation treatment obtained in Comparative Example 1.

[Figure 4] This is a graph showing the quantity of oxidized LDL in CCU serum and the serum of normal persons that had undergone oxidation treatment obtained in Example 6.

[Figure 5] This is a graph showing the quantity of oxidized LDL in V-50 treated samples of CCU serum and serum of normal persons that had been treated with 2,2'-azobis(2-amidinopropane) · dihydrochloride (V-50) obtained in Example 7.

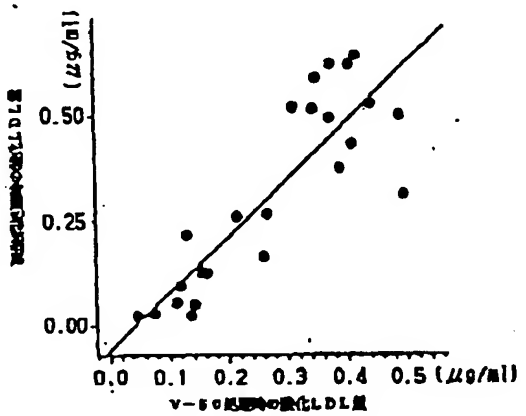
[Figure 6] This is a graph showing the relationship between the quantity of oxidized LDL in samples that had undergone oxidation treatment obtained when treatment was performed with copper sulfate and the quantity of oxidized LDL in samples that undergo oxidation treatment when treated with V-50 obtained in Example 7.

[Translator's Note: not provided with the Japanese Patent supplied us.]

[Explanation of the Symbols in the Figures]

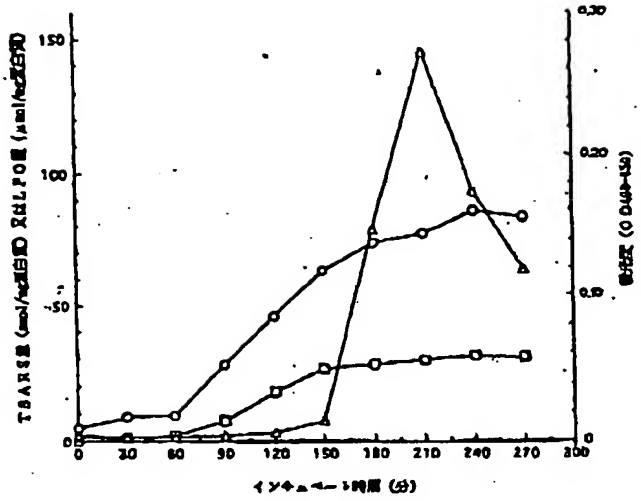
In Figure 1, Δ indicates the absorbance originating from oxidized LDL obtained for the specified test materials, O indicates the quantity of thiobarbituric acid reaction substance (TBARS) obtained for the specified test materials and □ indicates the quantity of lipid peroxide (LPO) obtained for the specified test materials. In Figure 2, Δ shows the results when serum originating from normal individuals was used and O and □ shows the result when CCU serum was used.

Figure 6



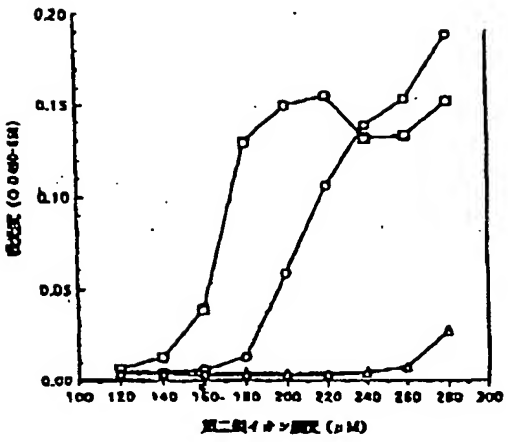
[vertical axis]: Quantity of oxidized LDL during copper sulfate treatment
[horizontal axis]: Quantity of oxidized LDL during V-50 treatment

Figure 1



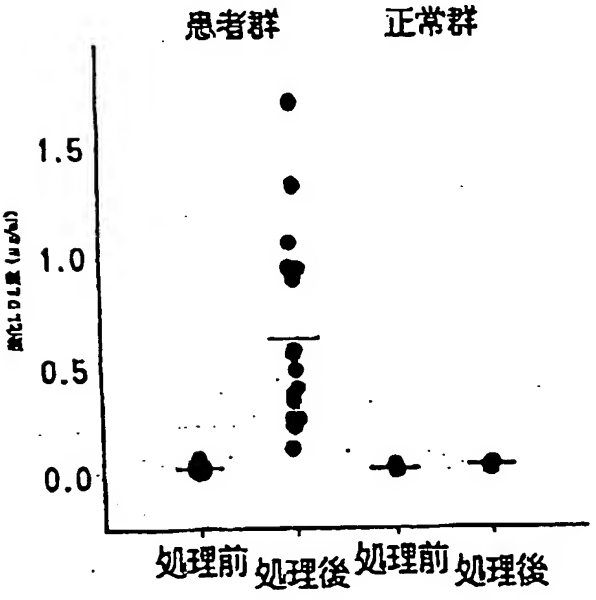
[left vertical axis]: Quantity of TBARS (nmol/mg protein) or quantity of LPO (nmol/mg protein)
[right vertical axis]: Absorbance (OD 490-650)
[horizontal axis]: Incubation time (minutes)

Figure 2



[vertical axis]: Absorbance (OD 490-650)
[horizontal axis]: Cuprous ion concentration (μM)

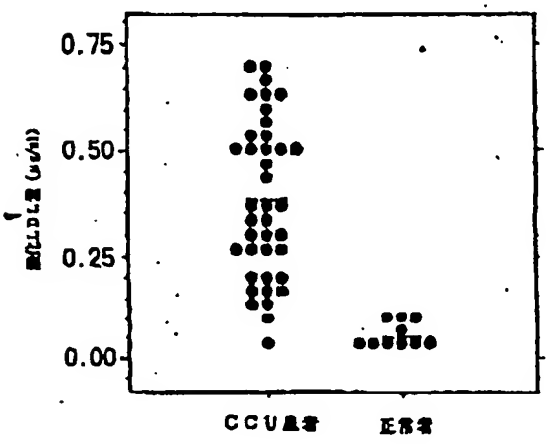
Figure 3



[vertical axis]: Quantity of oxidized LDL ($\mu g/ml$)
[horizontal axis]: Before treatment; After treatment; Before treatment; After treatment

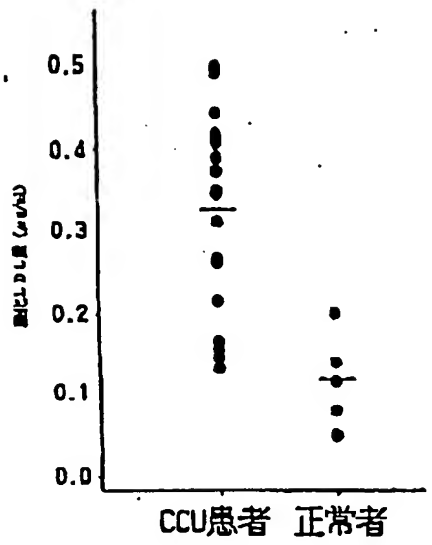
[at top of graph]: Patient group; Normal group

Figure 4



[vertical axis]: Quantity of oxidized LDL ($\mu g/ml$)
[horizontal axis]: CCU patients; Normal individuals

Figure 5



[vertical axis]: Quantity of oxidized LDL (μg/ml)
[horizontal axis]: CCU patients, Normal individuals